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Cancer Research

Amphiregulin Is a Critical Downstream Effector of Estrogen Signaling in ERα-Positive Breast Cancer

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Abstract

Estrogen stimulation promotes epithelial cell proliferation in estrogen receptor (ERα)-positive breast cancer. Many ERα target genes have been enumerated, but the identities of the key effectors mediating the estrogen signal remain obscure. During mouse mammary gland development, the estrogen growth factor receptor (EGFR) ligand amphiregulin acts as an important stage-specific effector of estrogen signaling. In this study, we investigated the role of amphiregulin in breast cancer cell proliferation using human tissue samples and tumor xenografts in mice. Amphiregulin was enriched in ERα-positive human breast tumor cells and required for estrogen-dependent growth of MCF7 tumor xenografts. Furthermore, amphiregulin levels were suppressed in patients treated with endocrine therapy. Suppression of EGF receptor signaling appeared necessary for the therapeutic response in this setting. Our findings implicate amphiregulin as a critical mediator of the estrogen response in ERα-positive breast cancer, emphasizing the importance of EGF receptor signaling in breast tumor pathogenesis and therapeutic response. *Cancer Res; 75(22); 1–9. ©2015 AACR.*

Introduction

Estrogen is an essential hormone for mammary gland development and is a key driver of proliferation during the development of estrogen receptor–positive ($ERα^+$) breast tumors. The actions of estrogen are primarily mediated by its receptor, the ERα transcription factor, which is required for mammary gland development (1). Microarray and chromatin immunoprecipitation experiments have identified several hundred estrogen-responsive genes in breast cancer cells (2, 3); however, among these targets, the identity of the key effectors of this proliferative signal in breast cancer remains unclear. A more detailed understanding of the mechanisms involved will provide insight into the processes driving $ERα^+$ breast tumor initiation and progression.

Analysis of human mammary glands demonstrated that it is the epithelial cells adjacent to $ERα^+$ cells (rather than the $ERα^+$ cells themselves) which enter the cell cycle following estrogen stimulation (4), implicating an estrogen-responsive paracrine growth factor in proliferation control. In the mouse, mammary gland development from ERα-deficient cells can be rescued by cotransplanting with wild-type mammary epithelial cells, supporting a role for a paracrine factor (5). We and others have previously reported that amphiregulin (AREG), a ligand of the estrogen growth factor receptor (EGFR), is induced during the proliferative phase of mouse pubertal mammary growth, where it is a direct transcriptional target of ERα (6, 7). Mammary glands of amphiregulin knockout mice have a striking defect in pubertal epithelial outgrowth but retain the ability to undergo differentiation during pregnancy, indicating a stage-specific requirement (8). This phenotype is rescued by cotransplantation of wild-type and $Areg^{-/-}$ mammary epithelial cells (6). Thus, amphiregulin appears to be a key mediator of estrogen action during normal mammary gland development.

Studies of human breast cancer cell lines indicate that amphiregulin is induced by estrogen treatment (9), and that its experimental overexpression can confer EGF signaling self-sufficiency (10), but whether endogenous amphiregulin plays an important role in the estrogen-dependent proliferation of human breast cancer cells remains unknown. In this study, we test the hypothesis that co-option of this key stage-specific mammary developmental pathway might be the primary driver of estrogen-dependent proliferation of $ERα^+$ human breast cancer cells.

Materials and Methods

Cell culture

MCF-7, T47D, and ZR751 were obtained from ATCC, and independently validated by STR profiling at our institution. These cell lines were cultured in DMEM (Cellgro) with 10% FBS (Hyclone). Suppression of *AREG* expression was achieved by lentiviral infection with two independent pLKO.1 constructs with the following sequences: shAREG-1, cactgccaagtcatagccata; shAREG-2, gaacgaaagaaacttcgacaa; or the empty vector control. For three-dimensional (3D) culture and *in vivo* experiments, FACS sorting was used to enrich for cells from shRNA-transduced pools which lacked cell surface amphiregulin.

Quantitative reverse transcriptase PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. One microgram of RNA was used for cDNA synthesis using the ImProm-II Reverse

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Transcriptase (Promega) in a 20-μL total reaction volume. Relative expression levels were determined by qPCR assays performed on a Bio-Rad IQ5 Multicolor Real-Time PCR Detection System using primers for AREG (5′-tgatcctcacagctgttgct-3′ and 5′-tggctatgacttggcagtga-3′), and GAPDH (5′-cccactcctccacctttgac-3′ and 5′-cataccaggaaatgagcttga-3′).

ELISA

The human amphiregulin DuoSet ELISA Development System (R&D Systems) was used to analyze amphiregulin levels according to the manufacturer's instructions, as previously described (11).

Tumor xenografts

All xenografts were performed in athymic mice and were approved by the Institutional Animal Use and Care Committee of the Albert Einstein College of Medicine (New York, NY). Two series of *AREG* knockdown experiments were performed. In the first, 14 nulliparous 5-week-old athymic mice were implanted with 0.72-mg 17β-estradiol 60-day release pellets, and injected orthotopically with 1×10^6 MCF7 cells in a 1:1 mixture of DMEM and Matrigel in the right (AREG knockdown: shAREG-2) or left (empty vector control) fourth inguinal mammary fat pad of each mouse. Tumor growth was monitored for 51 days. The second series was performed identically, except 12 mice were used and monitored for 44 days.

Immunohistochemistry

Breast tumor tissue microarrays (TMA) were provided by The Ohio State University's Human Genetics Sample Bank. Slides were dewaxed in histoclear and rehydrated by serial incubations in 100% to 70% ethanol. Slides were rinsed with water and then with TBS. Antigen retrieval was performed by incubation of slides in a steamer for 20 minutes in a preboiled solution of 10 mmol/L sodium citrate (pH 6.0). Slides were washed in TBS and incubated for 30 minutes in a solution of 2% hydrogen peroxide in 1:1 methanol:PBS. Slides were washed in TBS, blocked (5% rabbit serum in PBS), and immunostained with goat anti-AREG antibody (15 mg/mL; AF262, R&D Systems) overnight at 4°C. Slides were washed five times in TBS, followed by incubation for 30 minutes at room temperature in a 1:300 dilution of biotinylated anti-goat IgG antibody (Vector Laboratories, Inc.). Samples were incubated for 30 minutes at room temperature in Vectastain Elite ABC-HRP, washed twice in TBS and developed using 3, 3′-diaminobenzidine (Vector Laboratories, Inc). Samples were washed with water and counterstained with hematoxylin, rinsed with water, dehydrated by serial ethanol washes to 100%, incubated in histoclear for 3 minutes, and mounted in Permount (Fisher Scientific). Amphiregulin staining intensity was assessed semiquantitatively using a three-point scale by two investigators working independently on blinded samples. Discordant scores were resolved by joint review. Proliferation was assessed using mouse anti-BrdU (Roche) at a 1:400 dilution.

3D culture proliferation assay

Three-dimensional laminin-rich extracellular matrix cultures were prepared by seeding of single cells on top of a thin layer of growth factor–reduced Matrigel (BD Biosciences) and the addition of a medium containing 5% Matrigel, as previously described (12, 13). The cell lines were seeded at a density of 1,000 cells/cm^2 for MCF7 and 625 cells/cm^2 for T47D and ZR751. Cells were seeded in DMEM supplemented with 1% charcoal/dextran-stripped FBS (Gemini Bioproducts), 0.292 mg/mL L-glutamine, 1× nonessential amino acids, 10.11 mg/mL sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL of streptomycin (Hyclone), and 6 ng/mL of human recombinant insulin (Calbiochem). Digital pictures of each well were taken and colony cross-sectional area was measured using ImageJ.

Microarray and clinical data

Gene expression profiles of breast cancer cell lines in 3D culture (12) are available from ArrayExpress (#E-TABM-244). Gene expression profiles (NCBI accession number: GSE5462) from a study of paired tumor core biopsies taken before and after 14 days of treatment with letrozole (14, 15) were examined for the expression of amphiregulin and other ERBB ligands and receptors. Of the 58 patients included in the study, response data were not available for 6 cases, resulting in a total of 52 paired samples included in our analysis.

Results

We examined the distribution of amphiregulin expression in 295 breast cancer patients (16), which revealed a striking enrichment of *AREG* mRNA expression in ERα$^+$ tumors (Fig. 1A). We further evaluated the quantitative relationship between *AREG* and ERα (*ESR1*) mRNA levels in 13 luminal human breast cancer cell lines grown in 3D culture (12). The highest levels of *AREG* are found in luminal cell lines with the highest levels of ERα expression (Fig. 1B). We confirmed the association between AREG and ERα expression in an independent cohort of 118 breast cancer patients by immunostaining for amphiregulin on tumor tissue microarrays (Fig. 1C). Analysis of AREG levels in 88 ERα$^+$ and 30 ERα$^-$ tumors showed that ERα$^+$ tumors most frequently express high levels of the amphiregulin protein ($P = 0.0194$). Representative examples of staining intensity are provided and additional sections can be seen in the Supplementary Materials and Methods.

As previously reported (9), we found that *AREG* mRNA is regulated by estrogen in MCF7 cells and extended this finding to T47D cells, an additional ERα$^+$ breast cancer cell line. AREG mRNA was induced by estradiol and is suppressed by ERα antagonists with distinct mechanisms of action such as 4-hydroxytamoxifen (OHT) and fulvestrant (ICI182,780) in MCF7 (Fig. 2A) and T47D (Fig. 2B) cells. Using ELISA, we found that production of soluble amphiregulin protein was increased upon estradiol treatment, and was suppressed by both ERα antagonists in MCF7 (Fig. 2C) and T47D (Fig. 2D) cells.

These data are consistent with *AREG* being a transcriptional target of ERα in both human breast tumors and breast cancer cell lines; however, the extent to which AREG, among hundreds of known ERα target genes (2, 3), is a key effector of ERα function remained unclear. To rigorously test the requirement for AREG in ERα-dependent proliferation, we used two shRNA constructs to establish pools of MCF7 cells with stable suppression of amphiregulin expression. Efficient knockdown of amphiregulin in these pools compared with the empty vector control (pLKO.1) was confirmed by both quantitative reverse transcription (qRT)-PCR (Fig. 3A) and ELISA (Fig. 3B) analysis. To evaluate the impact of amphiregulin depletion on the proliferative response to estrogen, we performed 3D culture experiments. The vector control (pLKO.1) MCF7 cell line exhibited a robust growth response to

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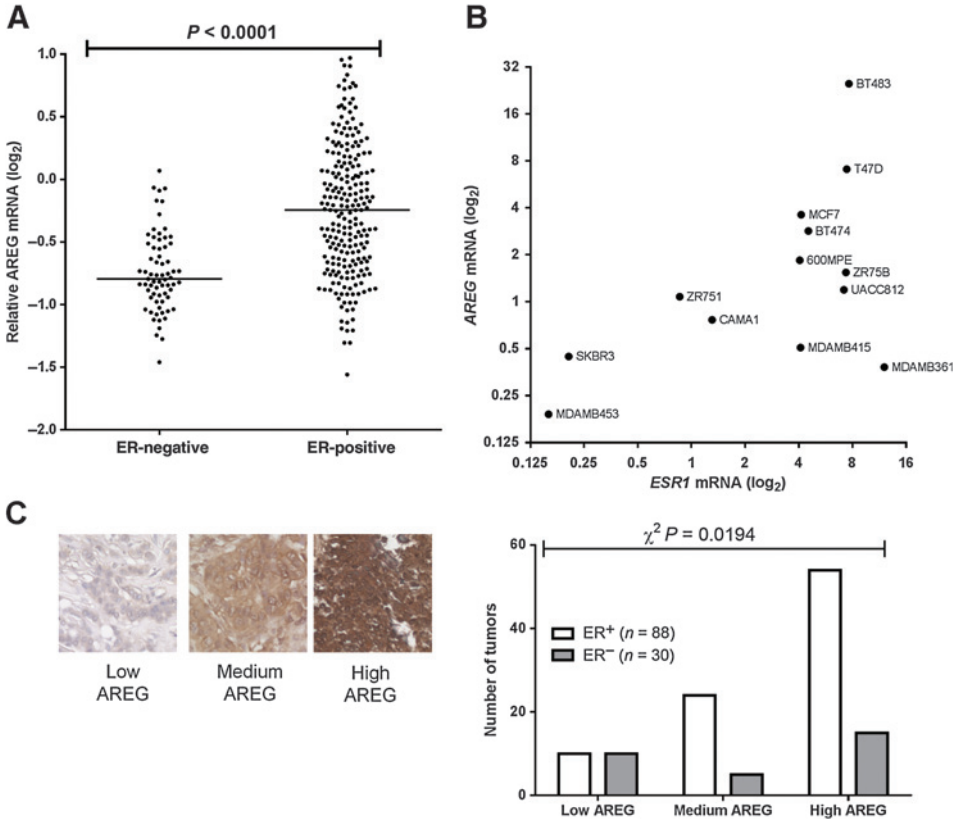

Figure 1.
Amphiregulin expression is enriched in ERα$^+$ human breast cancer. A, gene expression microarray analysis of *AREG* expression in 295 human breast tumors stratified by estrogen receptor status. B, comparison of *AREG* and *ESR1* mRNA levels in 13 human luminal-subtype breast cancer cell lines. C, semiquantitative immunohistochemical analysis of AREG protein expression in 118 human breast cancer cases in tumor tissue microarray format ($n = 88$ ER$^+$, $n = 30$ ER$^-$). *P* value was determined using χ^2 test. Representative images of staining intensities are shown.

estrogen, while neither shRNA-transduced subline responded (Fig. 3C). To test whether the requirement for amphiregulin for growth in 3D culture was a general feature of ER$^+$ breast cancer cell lines, we suppressed amphiregulin expression in two additional lines, ZR751 and T47D (Fig. 3D). In both cases, the vector control lines grew well in response to estrogen, while the shAREG-transduced pools responded weakly or not at all (Fig. 3E and F).

To ascertain the importance of estrogen-dependent induction of amphiregulin *in vivo*, control and knockdown MCF7 cells were injected into mammary fat pads of athymic mice implanted with slow-release estrogen pellets. The two shRNA lines were evaluated on different dates and the contemporaneous vector control is shown in each case (Fig. 4A). Tumors in which amphiregulin was knocked down grew significantly more slowly than control tumors ($P < 0.05$ at day 17 and beyond, black curves, and day 27 and beyond, gray curves). Sustained suppression of AREG expression was confirmed by ELISA analysis of tumor lysates at the endpoint of the experiment which included the shAREG#2 cell line (Fig. 4B). These data indicate that, among ERα target genes, amphiregulin expression is necessary for the robust growth of MCF7 tumors *in vivo*.

To determine the extent to which these *in vitro* and *in vivo* findings could be generalized to human breast cancer cases, we examined the change in expression of amphiregulin mRNA following a 2-week treatment with the aromatase inhibitor, letrozole, in 52 postmenopausal breast cancer cases previously described by Miller and colleagues (14, 15). Response to treatment was assessed as a reduction in tumor volume of greater than 50%, as measured by ultrasonography, after 3 months of neoadjuvant letrozole. Figure 5A shows the fold change in amphiregulin from baseline following 2 weeks of letrozole treatment. The majority of cases (41/52) had a substantial reduction in amphiregulin expression levels, a finding observed in both responders and nonresponders. These data suggest that amphiregulin expression is regulated by ERα activity in human breast tumors.

It was interesting to see that some of these tumors had a substantial reduction in amphiregulin levels yet failed to achieve a 50% reduction in tumor volume. To determine whether the ERBB pathways might remain active in the nonresponding tumors even when amphiregulin mRNA was suppressed, we examined differences in expression levels of all ERBB family receptors and ligands between responders and nonresponders in the post-letrozole treatment samples. Nonresponding tumors had consistently and significantly higher expression levels of the genes encoding the ERBB2 receptor and the ligands TGFα, epiregulin, neuregulin 1, and neuregulin 2 (Fig. 5B). These data suggest that tumors in which the primary estrogen-responsive ERBB family ligand, amphiregulin, is suppressed by endocrine therapy but which express alternate ligands and/or receptors which can activate these same signaling pathways may escape the growth-suppressive effects of endocrine therapies. To determine whether some of these genes might be acting coordinately, we examined their expression in the individual tumors (Fig. 5C). Although statistically significant (Fig. 5B), the differences in epiregulin and TGFα expression between responders and nonresponders were not very striking. In contrast, the nonresponders were enriched for tumors with elevated levels of ERBB2, NRG1, and NRG2, suggesting that tumors in which elevated expressions of these ligands (whose

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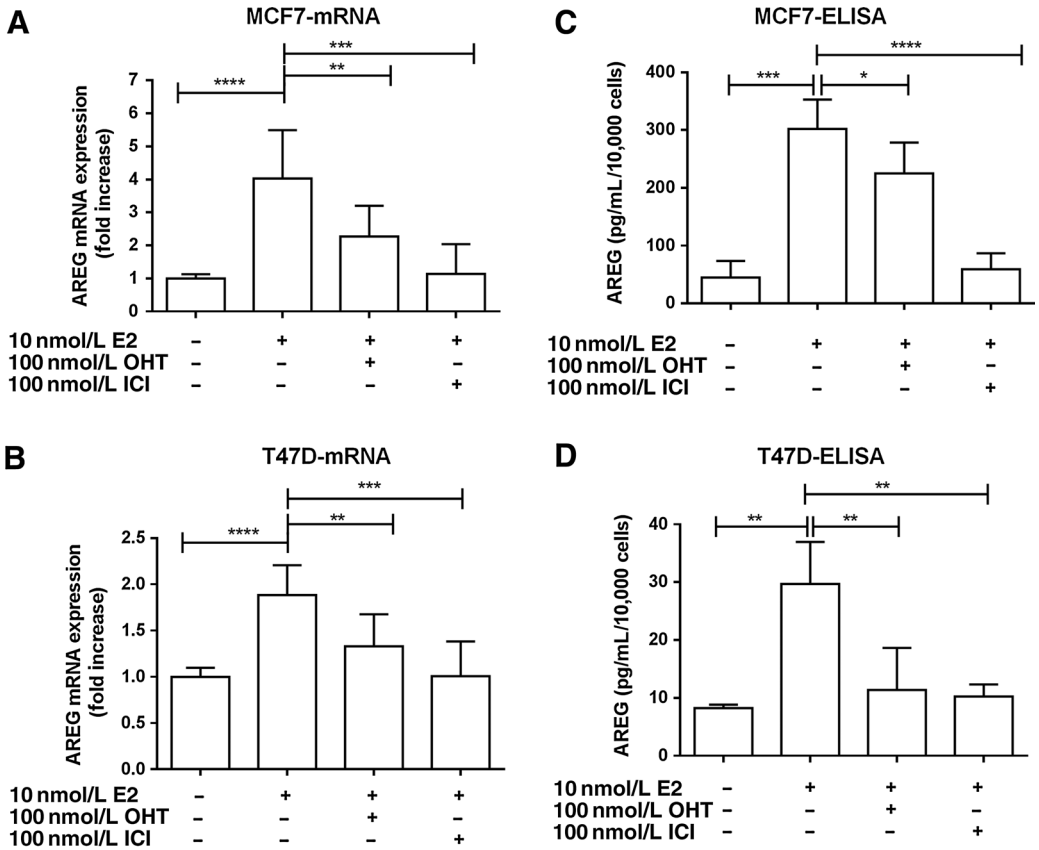

Figure 2.
Amphiregulin expression is induced by estrogen and suppressed by ERα antagonists. A, quantitative RT-PCR analysis of *AREG* mRNA levels in MCF7 cells treated with estrogen (E2) alone, or supplemented with 4-hydroxytamoxifen (OHT) or fulvestrant (ICI) at the indicated concentrations. B, quantitative RT-PCR analysis of *AREG* mRNA levels in T47D cells treated as described in A. C, MCF7 and D, T47D, ELISA analysis of soluble AREG protein production by cell lines treated as indicated in A. Error bars represent SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

receptors heterodimerize with ERBB2) may be less likely to respond to endocrine therapy.

Discussion

In this study, we found that amphiregulin expression is frequently associated with estrogen receptor positivity in human breast tumors and cell lines, and that estrogen-dependent amphiregulin expression is necessary for the growth of MCF7 xenografts *in vivo*, and for the estrogen-responsive growth of several ER^+ breast cancer cell lines in 3D culture. These data indicate that the co-option of this stage-specific mammary developmental pathway may be a key feature of $ER\alpha^+$ human breast cancer. The clinical relevance of these experimental findings is supported by the strong suppression of amphiregulin expression observed in a large cohort of breast cancer patients who received a neoadjuvant endocrine therapy, and by the demonstration that several tumors that did not respond to treatment had alternate, likely estrogen-independent, mechanisms of activating ERBB signaling pathways.

Several mechanisms have been proposed to explain the proliferative response to estrogen in $ER\alpha^+$ breast cancer cells, including the upregulation of c-Myc (17), Cyclin D1 (18), c-Myb (19), GREB1 (20), interaction with RSK (21), and activation of Cyclin E/cdk2 (22). Each of these potential mechanisms relies on cell-autonomous effects of the estrogen receptor, yet in the normal human breast, it is not the $ER\alpha^+$ cells, but the cells immediately adjacent to them that proliferate (4), suggesting that a paracrine effector is also involved. Similarly, in $ER\alpha^+$ breast tumors, significant proportions of the neoplastic cells can lack ERα expression, yet these tumors often respond well to endocrine therapy (23). Together, these findings indicate that estrogen has both cell-autonomous and non–cell autonomous effects during mammary gland development and in breast cancer, and that autocrine and paracrine mechanisms, which likely include amphiregulin, may play an important role in both settings.

Autocrine amphiregulin expression has been implicated in the growth of inflammatory and other ER^- breast cancer cell lines in culture (24, 25); however, despite the reported importance of amphiregulin as an estrogen effector during mouse mammary gland development (6), and evidence that amphiregulin is also regulated by estrogen in human breast cancer cell lines (9), the actual contribution of amphiregulin/EGFR to estrogen-dependent human breast cancer initiation and progression has not received widespread attention. This lack of attention may reflect, in part, the disappointment that initial trials of EGFR inhibition in breast cancer were not very successful (summarized in ref. 26), although the understanding of the biology of EGFR was less advanced at that time and there was often little attempt at rational

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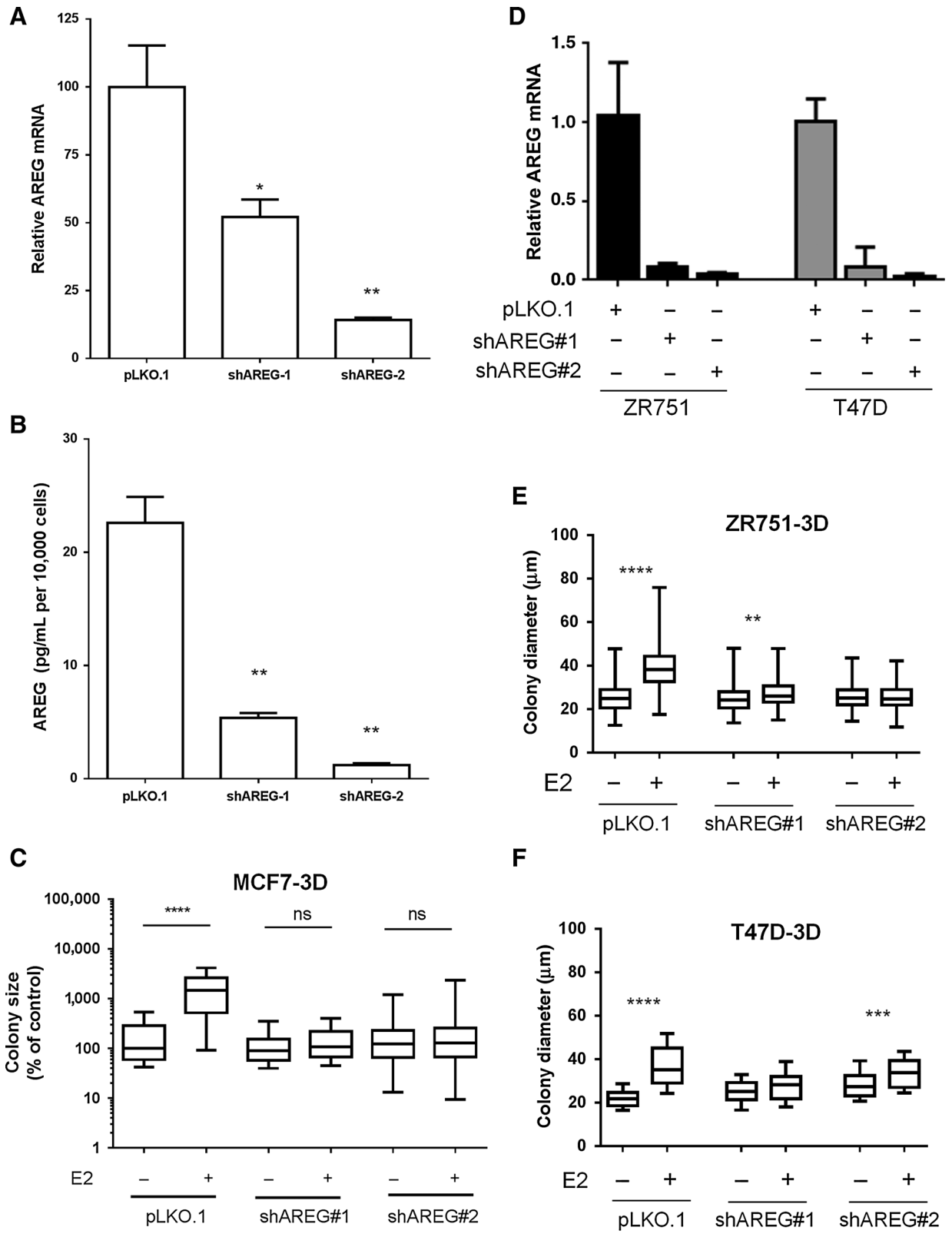

Figure 3.

shRNA-mediated knockdown of amphiregulin strongly attenuates ERα$^+$ breast cancer cell line growth *in vitro*. A, qRT-PCR analysis of *AREG* knockdown using two independent shRNA constructs. B, decreased AREG soluble protein production in shRNA transductants, detected by ELISA. (*Continued on the following page.*)

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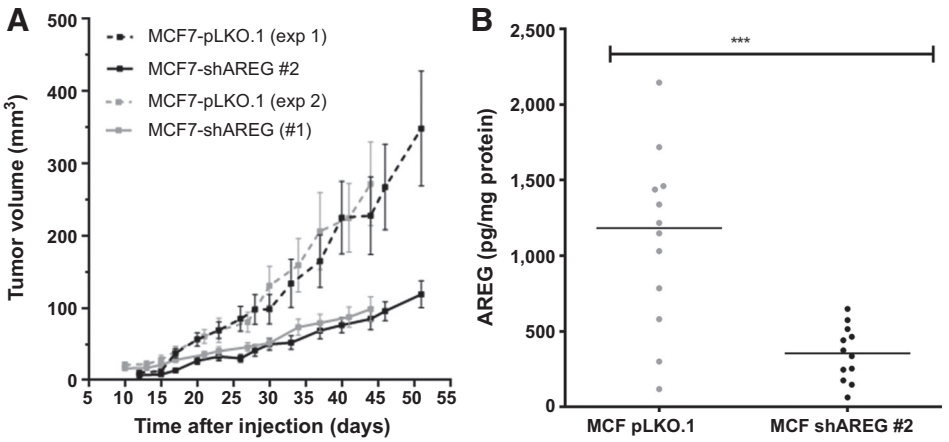

Figure 4.
shRNA-mediated suppression of AREG attenuates estrogen-responsive MCF7 xenograft growth *in vivo*. A, comparison of the xenograft growth rates of MCF7-shAREG and vector control tumors ($n = 14$ tumors per group for black curves, 12 tumors per group for gray curves). B, ELISA analysis of human amphiregulin from the xenografted tumors harvested 51 days after injection from one series of *in vivo* experiments (black curves in A).

patient selection in these studies. The previously conducted clinical trials have typically addressed the combination of an EGFR inhibitor with an endocrine agent, while our data support the argument that endocrine therapies may act by suppressing the expression of the EGFR ligand, amphiregulin, and may thus function indirectly as EGFR pathway inhibitors. If these anti-EGFR and anti-estrogen therapies are indeed impinging on the same key pathway, overly simplistic conclusions from combination clinical trial data may merit closer examination.

For example, a phase II trial in the neoadjuvant setting of 206 women with ERα$^+$ tumors, randomized to anastrazole alone or anastrazole plus gefitinib (i.e., aromatase inhibitor ± EGFR inhibitor) showed that gefitinib added no additional benefit to aromatase inhibition (27), which could suggest that either gefitinib has no activity in breast cancer or that it targets the same pathway as aromatase inhibitors. Studies with single-agent gefitinib arms indicate that a substantial proportion of ERα$^+$ tumors exhibit either a molecular or clinical response to EGFR inhibition, particularly among patients not heavily pretreated with other agents. For example, Guix and colleagues treated 41 women preoperatively with the EGFR inhibitor, erlotinib, and saw a significant downregulation of Ki67 levels in ERα$^+$ but not HER2$^+$ or triple-negative (ERα$^-$ PR$^-$ HER2$^-$) breast cancer (28). Polychronis and colleagues randomized 54 women with ER$^+$ breast tumors to gefitinib with or without anastrazole and clearly showed that gefitinib alone significantly downregulated both tumor cell proliferation and tumor bulk when given for 4 to 6 weeks preoperatively (29). In ERα$^+$ metastatic breast cancer, adding gefitinib to anastrazole significantly increased progression-free survival (PFS) compared with anastrazole alone (median PFS 22 vs. 14.7 months) with the benefit being particularly pronounced in women who had not previously received endocrine therapy (median PFS 20.2 vs. 8.4 months; ref. 30). In patients with ERα$^+$ tumors with acquired resistance to tamoxifen, gefitinib was associated with a 53.6% clinical benefit rate, significantly more than the 11.5% rate observed in ERα$^-$ tumors in the same trial (31); however, a trial in which the ERα$^+$ tumors had already acquired resistance to both tamoxifen and an aromatase inhibitor showed no benefit of gefitinib (32). Similarly, a study by Baselga and colleagues in advanced breast cancer patients with 1 to 2 prior chemotherapy regimens did not show benefit for gefitinib (33); however, this trial involved a smaller number of patients ($n = 31$), less than half of whom actually expressed EGFR in their tumor. Taken together, although EGFR inhibitors have not proven to be a panacea for breast cancer, several lines of evidence suggest that EGFR plays a role in ERα$^+$ breast cancer, at least up to the stage of treatment resistance, and is likely functioning downstream of ERα. The ERα target gene and EGFR ligand, amphiregulin, is an attractive candidate to link these pathways.

If amphiregulin/EGFR signaling contributes to a significant proportion of ERα$^+$ breast cancer cases, the lack of enduring responses to gefitinib and erlotinib requires some explanation. We speculate that achieving complete and sustained inhibition of wild-type EGFR using these single agents *in vivo* is difficult; thus, many of these tumors may be dependent on the EGFR pathway, yet at the same time insensitive to EGFR inhibitors. A key aspect of this pathway is the extent to which ligand binding by a minor fraction of receptors can yield a robust pathway activation due to the stoichiometry between receptors and the large number of downstream signaling intermediates, and the signaling amplification that takes place at each step of the pathway. Chen and colleagues have reported an advanced mathematical model (34) describing the relationship between the various ERBB receptors and downstream intermediates and computed the rates of signal propagation via these intermediates at various levels of ERBB activation (using either EGF or heregulin across a concentration range of several logs). Importantly, the model equations were validated against biologic experiments, providing a detailed quantitative analysis of all of the key parameters in several cancer cell lines. As one might expect, activation of the EGFR itself by EGF

(*Continued.*) C, quantification of colony cross-sectional area in 3D Matrigel cultures after 14 days showing that the proliferative response to estradiol observed in the control samples (left) is abrogated upon shRNA-mediated suppression of AREG expression (right). The graph represents quantification of three independent experiments, each normalized to the median colony size in the control cultures at the time of quantification. D, qRT-PCR analysis of AREG mRNA levels in ZR751 and T47D cell lines transduced with shAREG constructs. E, quantification of colony size (diameter) in 3D culture of ZR751 vector control and shAREG cell lines in the presence and absence of estrogen. F, quantification of colony size (diameter) in 3D culture of T47D vector control and shAREG cell lines in the presence and absence of estrogen.

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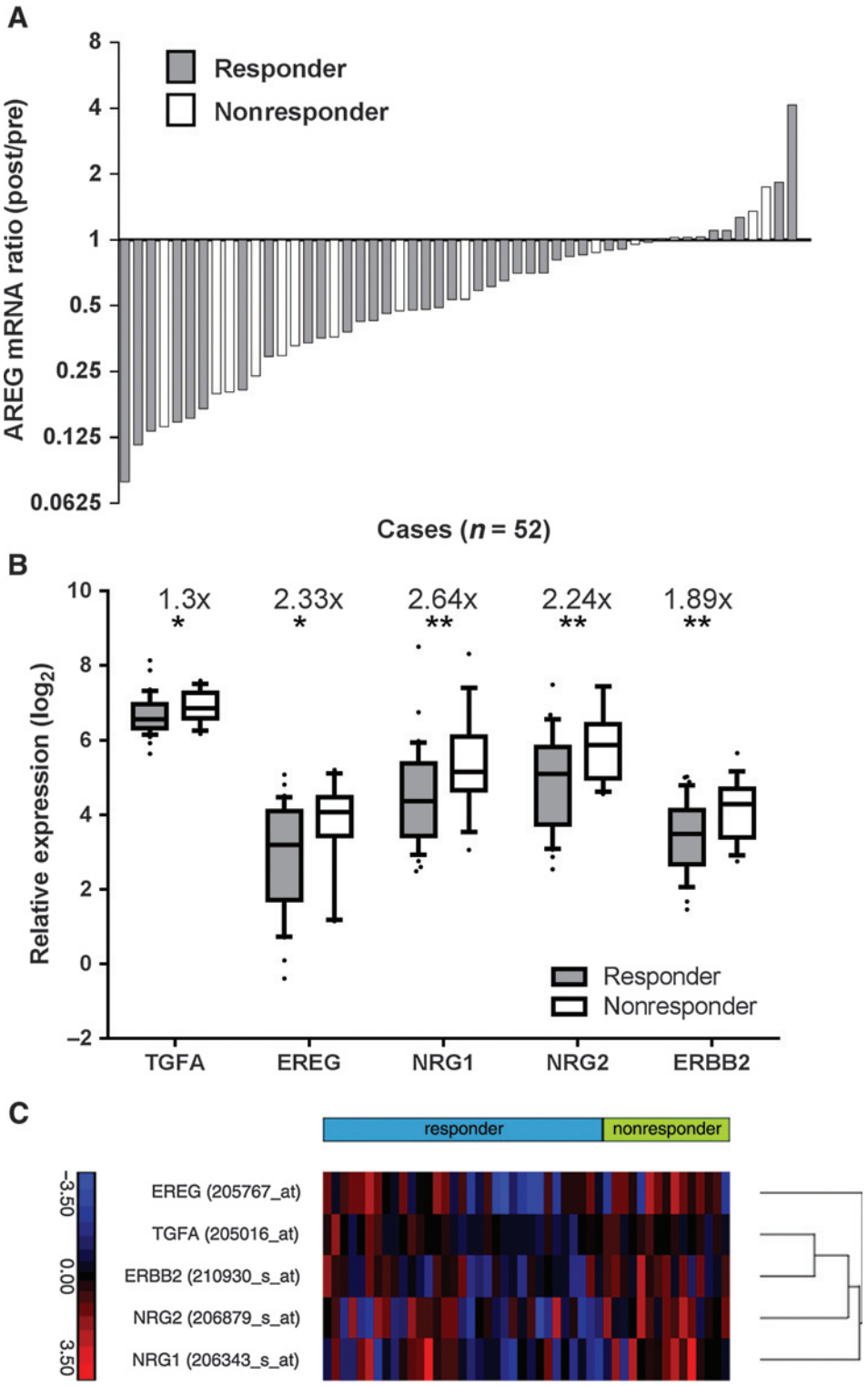

Figure 5.
Amphiregulin expression is strongly suppressed following neoadjuvant letrozole treatment of human breast cancer patients. A, amphiregulin mRNA analysis by gene expression microarray showing suppression of *AREG* levels following 2 weeks of letrozole treatment. The data represent the fold-change in *AREG* mRNA between the pretreatment and 14-day biopsy specimens. B, mRNA analysis of selected ERBB family receptors and ligands in 14-day biopsy specimens, stratified by ultrasonographically evaluated response after 90 days of neoadjuvant letrozole treatment. The between-group fold-difference in expression is indicated in each case, $P \leq 0.05$; **, $P \leq 0.01$. The following genes were also assessed and were not significantly different between the groups: *AREG*, *BTC*, *HBEGF*, *NRG3*, *NRG4*, *EGFR*, *ERBB3*, and *ERBB4*. C, heatmap of relative expression of each of the genes in B in each of the tumors. Levels of each gene were normalized to their average level across the population.

was substantially governed by the concentration of the ligand and by the enzymology of the receptor. However, examining the propagation of signal through the network reveals a substantial departure from linearity. In one example, a 50-fold reduction in the EGF stimulus (5 nmol/L to 0.1 nmol/L) resulted in an approximately 95% reduction in pEGFR while only reducing pMAPK by half and leaving pAkt essentially unchanged. In a gefitinib or erlotinib-treated tumor under steady-state conditions, the sub–10-minute half-life of the receptor–inhibitor complex (35), the high local concentrations of ERBB ligands (36, 37), and

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the capacity of the cellular signal transduction machinery to amplify small transient signals from occasionally uninhibited receptors at the cell surface, may mean that even 95% inhibition of the EGFR might not be sufficient to inhibit this pathway to an extent necessary to elicit a sustained tumor response *in vivo*. Experimental evidence indicates that the responsiveness of cancer cell lines to many inhibitors is highly regulated by the local concentration of receptor tyrosine kinase ligands (38). Thus, using higher-affinity EGFR inhibitors (35) or approaches to reduce ERBB family ligand bioavailability, such as using endocrine therapy to block amphiregulin induction or ADAM10/17 inhibitors to prevent ERBB ligand shedding (24), may have the potential to increase EGFR inhibitor effectiveness in this patient population.

In the clinic, almost half of patients with advanced ERα$^+$ tumors fail to respond to tamoxifen in the first-line setting and, of the patients who respond initially, all will subsequently progress to endocrine resistance (39). Elevated ERBB signaling activity has been associated with endocrine resistance in the clinic. For example, ERα$^+$ tumors that express high levels of the EGFR ligand, TGFα, tend to be tamoxifen nonresponsive (40). Our observations on the downregulation of AREG expression following letrozole treatment (Fig. 5A), and on the frequency of expression of ERBB signaling pathway activators in tumors not responding to letrozole (Fig. 5B and C), are consistent with a role for ERBB signaling generally in the proliferation of ERα$^+$ breast tumor cells and with our hypothesis that endocrine therapy-induced suppression of amphiregulin substantially contributes to the efficacy of these drugs. Interestingly, an *in vitro* MCF7 model selected for spontaneous resistance to aromatase inhibitors was found to have upregulated amphiregulin expression and to be amphiregulin dependent (41). Clearly, from our analysis of amphiregulin mRNA (Fig. 1A) and protein (Fig. 1C) levels in ERα$^-$ tumors, the estrogen receptor is not the sole regulator of amphiregulin expression. Identifying these alternate routes to amphiregulin expression may provide insight into the mechanisms driving amphiregulin expression in endocrine-resistant breast tumors.

In conclusion, we have implicated amphiregulin as a key effector of estrogen receptor activity during breast cancer growth *in vivo*. In light of our experimental data, we believe that the issue of EGFR signaling in ERα$^+$ breast tumors merits renewed attention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.A. Kenny
Development of methodology: E.C. Jenkins, E. Aranda
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.A. Peterson, E.C. Jenkins, K.A. Lofgren, N. Chandiramani, H. Liu, M. Barnett, P.A. Kenny
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.A. Peterson, E.C. Jenkins, K.A. Lofgren, E. Aranda, M. Barnett, P.A. Kenny
Writing, review, and/or revision of the manuscript: E.A. Peterson, E.C. Jenkins, K.A. Lofgren, H. Liu, P.A. Kenny
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Liu
Study supervision: P.A. Kenny

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